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BAC library development for allotetraploid *Leymus* (Triticeae) wildryes enable comparative genetic analysis of *lax-barrenstalk1* orthogene sequences and growth habit QTLs

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ABSTRACT

Tall-caespitose basin wildrye (*Leymus cinereus*) and rhizomatous creeping wildrye (*Leymus triticoides*) are perennial Triticeae relatives of wheat and barley. Quantitative trait loci (QTLs) controlling rhizome proliferation were previously detected on homoeologous regions of LG3a and LG3b in two full-sib families derived from allotetraploid hybrids. Triticeae homoeologous group 3 aligns to rice chromosome 1, which contains the rice *lax* panicle and maize *barrenstalk1* orthogene responsible for induction of axillary branch meristems, but this gene has not been mapped or sequenced in Triticeae. We developed bacterial artificial chromosome (BAC) libraries representing 6.1 haploid equivalents of the tetraploid *Leymus* genome (10.7 Mb). Overgo probes designed from the *lax-barrenstalk1* orthogene hybridized to 12 *Leymus* BAC clones. Deduced amino-acid sequences from seven BAC clones were highly conserved with the rice, maize, and sorghum *lax-barrenstalk1* orthogenes. Gene specific primers designed from two of the most divergent BAC clones map to homoeologous regions of *Leymus* LG3a and LG3b and align with the *lax-barrenstalk1* orthogene on rice 1L. Comparisons of genomic DNA sequences revealed two other conserved regions surrounding the *Leymus* LG3a, rice, and sorghum *lax-barrenstalk1* ortholoci, and one of these regions was also present in maize and *Leymus* LG3b sequences. Comparisons of *Leymus* LG3a and LG3b *lax-barrenstalk1* coding sequences and flanking genomic regions elucidate molecular differences between subgenomes.

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1. Introduction

The polyploid genus *Leymus* comprises about 30 perennial Triticeae grass species from diverse regions of the northern and southern hemisphere. *Leymus* wildryes and other perennial Triticeae grasses provide forage and habitat for livestock and wildlife; mitigate atmospheric dust, landslides, and soil erosion; combat weed invasions; and enhance economically important recreational activities throughout vast regions of the western United States and other dry temperate parts of the World. *Leymus* wildryes are also perennial relatives of several major cereal crops including wheat and barley. Wheat-*Leymus* chromosome introgression lines [1,2] also display traits such as biological nitrification inhibition [3] and other potentially useful traits.

Tall-caespitose basin wildrye (*Leymus cinereus*) and strongly rhizomatous creeping wildrye (*Leymus triticoides*) are two of the largest native grasses in western North America. Basin wildrye in particular is an unusually tall grass, exceeding 2 or 3 m in some environments [4–6]. Although basin wildrye is tolerant of moderately saline soils [7–10], creeping wildrye is particularly well adapted to saline meadows and has potential as a saline biomass crop [11,12]. In contrast, creeping wildrye is shorter but displays aggressive rhizome growth and a stronger propensity to form new tillers compared to basin wildrye [4,13]. Interspecific creeping × basin wildrye hybrids show a combination of traits including tall stature and rhizome branches that may also be useful in breeding regionally adapted perennial biomass crops [14].

Tetraploid creeping × basin wildrye hybrids have been used to construct molecular genetic maps [15], isolate and map EST

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markers [16,17] and identify markers or QTLs associated with a number of functionally important traits in two full-sib families, TTC1 and TTC2, derived from crosses of two *L. triticoides* (T) × *L. cinereus* (C) hybrids (TC1 and TC2) backcrossed to a *L. triticoides* tester plant (T-tester). Some of the traits evaluated include plant height, growth habit, and flowering characteristics [4]; fiber, protein, and mineral content traits [18]; and seed shattering [19]. Moreover, wheat-*Leymus* chromosome addition lines [1,20,21] provide a tertiary wheat gene pool for functionally important traits such as resistance to *Fusarium* head blight [21,22] and inhibition of biological nitrification [3]. The wheat-*Leymus* chromosome addition lines have been partially characterized using *Leymus* EST markers mapped to homoeologous group 3 [16].

Leymus EST libraries have provided a useful source of markers and candidate gene sequences [16,17], but these libraries are incomplete in the sense that not all genes are represented, coding sequences are typically incomplete, and the sequences do not contain functionally important upstream or downstream regulatory elements. In many cases, DNA polymorphisms responsible for functional trait variation are not represented in the EST libraries, which can encumber mapping and genetic analysis of targeted genome regions. Thus, more fully representational genomic DNA libraries are an essential resource for functional gene discovery research and applied plant improvement [23]. Representational BAC libraries are now available for most of the major cereals and the *Festuca–Lolium* grass complex [24,25] and perhaps switchgrass (no published reports available), but relatively few resources are available for gene and trait discovery research in large-statured, long-lived grasses.

Traits of particular interest in the creeping × basin wildrye hybrids, and other perennial grasses, are the caespitose and rhizomatous growth habits. Quantitative trait loci (QTLs) controlling the distance of rhizome spreading were detected on homoeologous regions of linkage groups (LG) 5Xm in the TTC2 family, LG6a of the TTC1 family, and homoeologous regions of LG3a and LG3b in both TTC1 and TTC2 families [4,16]. The coincidence of rhizome QTLs on Triticeae homoeologous group 3, in both families, provides evidence of one or more important gene factors controlling rhizome proliferation in this region. Only one other QTL controlling the distance of rhizome spreading has been identified on linkage group C in crosses of *Sorghum bicolor* and *Sorghum propinquum* [26], but otherwise there is very little known about the genetic control of rhizome proliferation because relatively few genetic mapping experiments have been done in perennial grasses. The *Oryza lax* panicle and maize (*Zea mays*) *barrenstalk1* orthogene is one of the few genes known to control the initiation and expression of axillary branch meristems in grasses [27,28]. The *lax-barrenstalk1* orthogenes are located on syntenic regions *Oryza* chromosome 1L and *Zea* 3L, encoding putative transcription factors containing a basic helix-loop-helix (bHLH) domains [27,28]. Rice 1L and Triticeae chromosome group 3L, including the *Leymus* LG3a and LG3b rhizome QTL region, are also highly conserved [16,29,30]. Markers flanking the *Sorghum* LG C (chromosome 1) rhizome QTL are syntenous and collinear with genes on *Oryza* chromosomes 3 and Triticeae homoeologous group 4 [31,32], whereas sequences on *Oryza* chromosome 1 including the *lax-barrenstalk1* orthogene are syntenous, and mostly collinear, from end to end with markers on *Sorghum* LG A (chromosome 3) [32,33]. Thus, the *lax-barrenstalk1* orthogene is predicted to align in or near the *Leymus* LG3a and LG3b rhizome QTLs, but not the *Sorghum* LG C rhizome QTL. However, the *lax-barrenstalk1* orthogene has not been reported in any cool-season grass despite the relatively large number of ESTs available in wheat, barley, and other Triticeae cereals. Thus, it was not previously possible to determine the exact map location and function of the *lax-barrenstalk1* orthogene, if present, in cool-season grasses such as the *Leymus* wildryes. Likewise, until the recent release of the *Sorghum* genome sequence [34], it was not previously determined if and where the *lax-barrenstalk1* orthogene was located in the *Sorghum* genome.

The primary objectives of this project were to construct representative genomic DNA libraries from a tetraploid hybrid of creeping wildrye and basin wildrye and isolate genomic DNA sequences of the *lax-barrenstalk1* orthogene including possible upstream and downstream regulatory regions which may be conserved among *Zea*, *Oryza*, and sorghum genome sequences. More than half of all *Leymus* species are tetraploids ($2n = 4x = 28$). The DNA content of the tetraploid *Leymus* is about 10.7×10^9 bp per haploid genome [35]. This is approximately midway between diploid barley (*Hordeum vulgare*) and hexaploid bread wheat (*Triticum aestivum*) Triticeae cereals [35], and approximately 24 times greater than the 430-Mb *Oryza* genome [36,37]. The *Leymus* genome was presumed to be allotetraploid formed by hybridization of the diploid *Psathyrostachys* and *Thinopyrum* Triticeae genera based on chromosome pairing [38–40]. The presence of *Psathyrostachys* (**Ns** genome) DNA in *Leymus* has been verified [41–43]. However, extensive testing has failed to detect *Thinopyrum*-specific DNA in *Leymus* [41]. Fluorescent in situ hybridization (FISH) mapping of dispersed retrotransposon-like repeats from North American *L. mollis* and northern European *L. arenarius* showed that these sequences were indiscriminately dispersed over all chromosomes of representative species of *Psathyrostachys* and *Leymus*, but not present in other Triticeae species. Based on these data, it was suggested that *Leymus* must be considered an autopolyploid $(\mathbf{Ns})_n$ or segmental allopolyploid combining different variations of the *Psathyrostachys* (**Ns**) genome [43]. Yet, we also know that hybrids of *L. cinereus* and *L. triticoides* showed disomic inheritance [15]. Thus, a secondary objective of this study was to compare sequence divergence and other possible sequence rearrangements between homoeologous loci showing disomic inheritance.

2. Materials and methods

2.1. BAC library construction

Young green leaves and stems were repeatedly harvested from source clones of the *L. triticoides* Acc641 × *L. cinereus* Acc636 TC2 hybrid [15] and then frozen in a −80 °C freezer. Two separate libraries were constructed by partial digestion of high-molecular weight *Leymus* DNA with *Bam*HI and *Mbo*I restriction enzymes, using previously described procedures [44–48]. Briefly, 70–100 g samples of frozen leaves were ground in liquid nitrogen, then transferred into 1× HB buffer and washed at least four times using the buffer. Resulting nuclei were embedded in 2.5 ml 1% low-melting-point (LMP) agarose plugs and incubated in lysis buffer (0.5 M EDTA; pH 9.0–9.3, 1% sodium lauryl sarcosine) containing proteinase K (0.3 mg/ml proteinase K) overnight at 50 °C, and then washed three times in ice-cold TE (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0) plus 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and stored in TE at 4 °C.

Each plug was cut into nine slices of equal size with a glass cover slip, followed by partial digestion with *Bam*HI and *Mbo*I restriction enzymes and analysis by pulsed-field gel electrophoresis (PFGE) using a CHEF DRIII system (Bio-Rad, Richmond, CA) to determine the optimal enzyme concentration. Large scale partial digestion was performed using 8–10 plugs, which were then size-selected on 1% PFGE with 0.5× TBE at 12.5 °C using 6 V/cm and 90-s switch time for 8 h, then 6 V/cm with a 50-s switch time for 7 h, and then 4 V/cm with a 5-s switch time for 3 h. DNA fractions ranging from 150 to 250 kb were excised from the gel, recovered by electo-elution, and dialyzed in 0.5× TE. The concentration of eluted DNA

was estimated on a 1% agarose gel using λ DNA as a standard. The size-selected DNA fragments were ligated with pECBAC1 vector DNA [46,47] in a 1:4 (vector:insert) ratio at 0.6–1.0 ng/μl. The vector DNA was isolated from overnight cultures by the alkaline lysis method, purified by cesium chloride (CsCl) gradient centrifugation, completely digested with *Bam*HI and *Mbo*I restriction enzymes, and dephosphorylated with calf intestinal alkaline phosphatase (Invitrogen, USA) [44,46]. The ligation reaction was performed in a volume of 100 μl with 2 units of T4 DNA ligase (Invitrogen, USA) at 16 °C for 19 h. Transformations were performed by mixing 1.0 μl of the ligation mixture and 20 μl of the *E. coli* ElectroMax DH10B cells in a cell-porator pulser 11613 (Gibco BRL, USA) at 2.5 kV. Transformed cells were incubated in 1 ml SOC medium [49] for 1 h at 37 °C and then grown on LB agar plates with X-gal and antibiotic. The average insert size from randomly selected blue colonies was analyzed by isolating DNA by alkaline lysis, digesting the DNA with *Not*I, and fractionating the resulting fragments on contour-clamped homogeneous electric field (CHEF) gels [44,46,47].

For long-term storage, the BAC clones were manually arrayed into 384-well microtiter plates, with each well containing about 65 μl of cell freezer storage medium with chloramphenicol [44,50]. The 384-well microtiter plates were incubated at 37 °C overnight to allow the clones to grow, replicated in three sets, and stored in freezers at −80 °C at two locations (Texas A&M University and the USDA-ARS Forage and Range Research Laboratory). The BAC libraries were printed robotically on 22.5-cm × 22.5-cm Hybond N+ membranes (GE Healthcare, Piscataway, NJ) in a 384-well 4 × 4 format using the GeneTAC™ G3 Robotic Workstation (Genomic Solutions, Inc., USA), with each clone double spotted in a unique orientation within each 4 × 4 grid. A total of six 384-well 4 × 4 arrays were printed on each filter. Thus, each filter has 36,864 spots for a total of 18,432 clones. The filters were processed according to a standard alkaline lysis method [44,50].

2.2. Initial sequencing of conserved Leymus lax-barrenstalk1 orthogene coding regions

Degenerate PCR primers (Table S1, Section 3 below) designed from the consensus sequence of the *O. lax* panicle AB115668 [27] and *Zea barrenstalk1* AY683001 [28] GenBank nucleotide sequences were tested for amplification of homologous DNA sequences from *Leymus*. Amplification of DNA sequences was performed in 50 μl volumes with 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each dNTP, 0.4 μM primers, 1 unit Jumpstart *Taq* DNA polymerase (Sigma, Saint Louis, MO), and plant genomic DNA. Thermal cycling was performed using 94 °C initial denaturation (1 min); 30 cycles of 94 °C denaturation (30 s), 60 °C annealing (30 s), and 72 °C extension (80 s); and 72 °C final extension (5 min). Amplification of *Leymus lax-barrenstalk1* transcript sequences was performed using 5′ RACE according to using commercially supplied reagents and protocols (BD Biosciences Clontech, Palo Alto, CA) using previously described cDNA preparations [51]. Amplification products were analyzed by agarose gel electrophoresis.

Cloning of PCR products was performed using a Qiagen PCR Cloning Kit (Valencia, CA). Cloning inserts were PCR amplified purified using the QuickStep 2 PCR Purification Kit (EdgeBio, Gaithersburg, MD). Both insert strands were sequenced using 1 μl of purified PCR amplicon (50 ng DNA), 1 μl of universal M13 primers (2 μM), 0.25 μl BigDye Terminator v3.1 DNA polymerase in 2.5× reaction buffer with an additional 2 μl of 5× reaction buffer, and 4 μl of water in a final reaction volume of 10 μl as recommended by commercial supplier (Applied Biosystems, Foster City, CA). Sequencing products were purified using Performa dye terminator removal (DTR) Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD), and fractionated on an ABI3730 (Applied Biosystems, Foster City, CA). Base calls were initially determined using Sequencing Analysis Software v 5.2 (Applied Biosystems). Base calls and chromatograms from complementary and/or overlapping reads (forward and reverse) were compared using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI).

2.3. Isolation of BAC clones containing Leymus lax-barrenstalk1 orthogene sequences

Overgo primers (Table S1, Section 3 below), were designed using the OVERGO 1.02i software [52] available at (http://www.mouse-genome.bcm.tmc.edu/). The 22 filters representing the two *Leymus* libraries were prehybridized in two sets, each with 250 ml of hybridization buffer (5× SSC [49], 0.5% SDS, 25 mM potassium phosphate buffer pH 6.5, 5× Denhardt's solution) for 2 h at 50 °C with gentle shaking. The overgo probe was labeled with [α-32 P]dCTP. Equimolar amounts of the overgo primer pairs were combined and denatured at 95 °C for 10 min and allowed to cool to room temperature and anneal. The reaction mixture was prepared using 200 ng of denatured overgo primer pairs combined with 24 μl of overgo labeling buffer [125 mM Tris–HCl, pH 8.0, 500 mM HEPES, pH 6.6, 12.5 mM $MgCl_2$, 0.175% (v/v) β-mercaptoethanol, 50 μM dATP, 50 μM dTTP, 50 μM dGTP], 2 U Klenow fragment, 4 μl [α-32 P]dCTP (6000 Ci/mmol) in a total volume of 50 μl. The reaction was incubated at 37 °C for 30 min, and was subjected to a Sephadex G-50 column to remove unincorporated nucleotides. To assist in accurately localizing each positive clone on the high-density clone filters, approximately 1 ng of labeled BAC vector pECBAC1 DNA was added to the labeled overgo probe. The probe was denatured by boiling for 10 min and immediately added to the prehybridized filters. The filters were hybridized at 50 °C for 18 h with gentle shaking. Filters were washed four times in 0.5× SSC, 0.1% SDS for at least 20 min each at 50 °C. The hybridized filters were exposed to X-ray film with intensifying screens at −80 °C. All BAC clones showing potentially significant hybridization with the overgo probe were tested for PCR amplification using gene specific primers, as described above, except that 1 μl of preculture (Section 2.4 below) was used as template.

2.4. BAC DNA isolations and sequencing

The DNA was isolated using the Qiagen Plasmid Midi Kit (Valencia, CA). Briefly, 5-ml precultures and 100-ml secondary cultures of selected BAC colonies were grown in 5 ml of LB with 20 μg/ml chloramphenicol at 37 °C with 250 rpm agitation for about 16 h. The 100-ml cultures were split into 50 ml tubes and centrifuged for 30 min at 3750 rpm (20 °C), which yield about 0.3–0.5 g of pellet per tube. Pellets were gently resuspended in 10 ml of P1 buffer with 100 μg/ml RNase and mixed with another 10 ml of clear P2 buffer solution by gently inverting tubes 4–6 times and incubated for 5 min at room temperature. The DNA solution was purified by adding 10 ml of chilled P3 buffer to each 30-ml subsamples, gently inverting tubes 4–6 times, removing floating precipitate, and passing both subsamples through one QIAfilter midi cartridge in 3 applications (about 10 min each). The DNA eluates were applied to equilibrated Qiagen-100 tips, which were then washed with two 10-ml applications of QC buffer. The DNA was eluted using five 1-ml aliquots of preheated (65°) QF buffer. The DNA was precipitated from the 5-ml QF solution by adding 3.5 ml of room temperature isopropanol, in 30-ml glass centrifuge tubes and pelleted at 15,000 g using an SS-34 rotor at 11,200 rpm for 30 min at 4 °C. The resulting pellets were washed down using 2 ml of ethanol and centrifuged again for 10 min. The concentrated pellets were dried for about 10 min and then dissolved using two 50-μl

washes of TE, which were then combined and dissolved overnight at 4 °C.

The BAC DNA templates were sequenced by using gene specific primers initially designed from sequences obtained by degenerate PCR and 5′ RACE experiments. Additional sequencing primers were obtained by sequential sequencing and primer synthesis, from both strands, downstream and upstream of the gene. Fluorescent dye terminator sequencing reactions were performed using 2 μl of purified BAC DNA (0.5–1 μg/μl), 6 μl of primer (20 μM), 8 μl of a 2.5× BigDye v3.2 reaction mix (Applied Biosystems), and 4 μl of water in a final reaction volume of 20 μl. Thermal cycling for the sequencing reactions was performed beginning with one 5 min step at 94 °C followed by 50 cycles of 94 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Sequencing products were subsequently fractionated and analyzed as described above.

2.5. Comparisons of divergent lax-barrenstalk1 orthogene sequences

Homology searches against the entire National Center for Biotechnology Information (NCBI) GenBank nucleotide collection and NCBI *Oryza sativa* subsp. *japonica* cultivar-group whole genome sequence (WGS) [36,37] available at (http://blast.ncbi.nlm.nih.gov/Blast.cgi), *Zea* BAC sequences on MaizeGDB [53] available at (http://www.Zeagdb.org), and *S. bicolor* genome sequence [34] available at Phytozome (http://www.phytozome.net/news.php) were performed using the Basic Local Alignment Tool (BLAST) [54] or Cross-species megaBLAST tool. Likewise, homology searches of translated open reading frames (ORFs) from *Leymus* BAC sequences against the entire NCBI GenBank translated protean sequence database were performed TBLASTX. Pairwise dot-plot and percent identity plots (PIPs) of the *Leymus* and *O. lax-barrenstalk1* genome regions were performed using (Advanced) PipMaker [55], available at http://pipmaker.bx.psu.edu/pipmaker/, with conserved sequence annotation generated using the sequence underlay option. Multiple-sequence alignments of putative homoeologous (intergenomic) and homologous (allelic) *Leymus lax-barrenstalk1*orthogene sequences was performed using ClustalW2 [56] available at http://www.ebi.ac.uk/Tools/clustalw2/index.html.

2.6. Linkage mapping

The 164-sib TTC1 and 170-sib TTC2 families, derived from crosses of the *L. triticoides* Acc641 × *L. cinereus* Acc636 TC1 and TC2 hybrids to a *L. triticoides* Acc641 T-tester plant were genotyped using locus-specific PCR primers designed from the *Leymus* BAC genomic DNA sequences (Table S1 and Section 3 below). The relative mobility of PCR amplicons was analyzed by capillary electrophoresis using GS500 LIZ internal size standard and ABI3730 genetic analyzer (PE Applied Biosystems Inc., Foster City, CA) and Genescan software (PE Applied Biosystems). The relative mobility of PCR amplicons was compared and classified, by genotype, using Genographer version 1.6.0 [57]. Polymorphic amplicons were initially assigned to one of 14 possible linkage groups in the TTC1 and/or TTC2 families [15,1] using the 'Create Groups Using a Map Node' and 'Assign Ungrouped Loci to Strongest Cross Link (SCL) Groups' functions of JoinMap 4.0 [58]. Consensus maps for LG3a and LG3b linkage groups [16] were recalculated by 'Regression Mapping' using only linkages with a recombination frequency smaller than 0.4, linkage LOD greater than 1.0, goodness-of-fit jump threshold of 5.0, ripples after each added locus, and Haldane's mapping function with a third round to force any remaining markers on the map. Alignments of *Leymus* LG3a and LG3b EST-SSR markers and homologous sequences on *Oryza* chromosome 1 were previously described [16], except for the addition of the putative *lax-barrenstalk1* ortholoci described below.

2.7. Reverse transcription PCR (RT-PCR)

RT-PCR was performed using previously described cDNA preparations [51] and the same PCR protocols described above (Section 2.2).

3. Results

3.1. BAC library size characteristics

A total of 313,728 *Bam*HI clones were arrayed in 817 384-well microplates and a sample of 93 clones displayed an average insert size of 155.0 kb (Fig. 1). Likewise, a total of 92,160 *Mbo*I clones were arrayed in 240 384-well microplates and a sample of 64 clones displayed an average insert size of 135.4 kb (Fig. 1). The combined libraries include 405,888 clones arrayed in 1057 384-well microplates with an average insert size of 150.5 kb. The *Bam*HI clones were double spotted on 17 filters and the *Mbo*I clones were double spotted on five filters.

3.2. Initial sequencing of conserved Leymus lax-barrenstalk1 orthogene coding regions

The *laxba1*.358f and *laxba1*.669r (Table S1) degenerate PCR primers, designed from the consensus sequence of the *O. lax* panicle AB115668 and *Zea barrenstalk1* AY683001 GenBank nucleotide sequences, amplified a single 270-bp product. Excluding 41 bp of primer sequence designed from the *lax-barrenstalk1* orthogene, the 229-bp *Leymus* amplicon (FJ373313) showed best homology to the *O. lax* and *Zea barrrenstalk1* orthogenes, when searched against the NCBI GenBank nucleotide collection, NCBI *Oryza* WGS, or *Zea* BAC sequences.

The GSP.392R (Table S1) 5′ RACE primer, designed from the *Leymus laxba1*.358f–*laxba1*.669r genomic DNA PCR amplicon (FJ373313) described above, amplified a 395-bp product. Excluding 41 bp of primer sequence, designed from the putative *lax-barrenstalk1* genomic DNA amplicon from *Leymus* (FJ373313), the remaining 370–bp *Leymus* transcript sequence (FJ373308) showed best homology to the *O. lax* and *Zea barrrenstalk1* orthogenes, when searched against the GenBank nucleotide collection, *Oryza* WGS, or *Zea* BAC sequences.

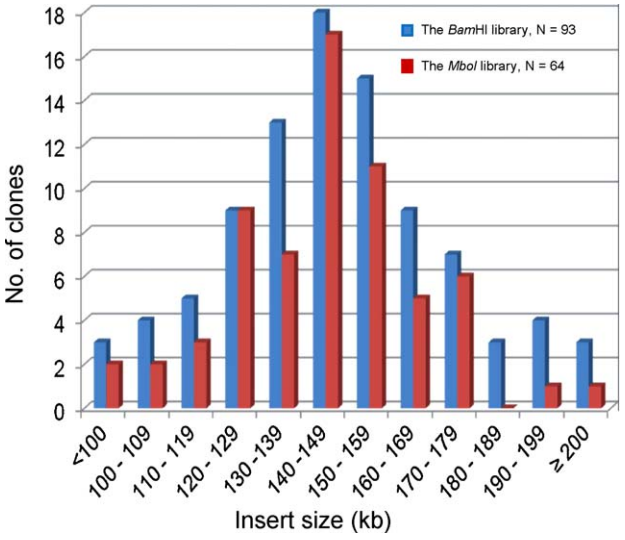

Fig. 1. Insert size distribution of clones randomly selected from the *Leymus Bam*HI and *Mbo*I BAC libraries. BAC DNA was isolated, digested with *Not*I to release the *Leymus* DNA inserts from the cloning vector, fractionated on pulsed-field gels, photographed and estimated in insert size.

3.3. BAC library screening

A total of twelve apparent hybridization signals with six *Bam*HI clones and six *Mbo*I clones were detected when the OG.057 + 43f and OG.057 + 81r overgo probe (Table S1) was hybridized to all 22 filters. In most cases, relatively dark double spot signals clearly distinguish hybridization of overgo probes versus hybridization of diluted vector to the overall 384-well 4 × 4 grid, which facilitated accurate identification of positive colonies. Seven of these 12 colonies showing hybridization with our overgo probe were confirmed by PCR using the GSP.057F and GSP.392R primer pair. All seven of these BAC clones were sequenced using the GSP.057F and GSP.566R primers (Table S1) and other primers designed by sequence walking upstream and downstream of the putative *Leymus lax-barrenstalk1* orthogene. Partial sequence analysis of non-coding regions from these seven BAC clones revealed two divergent sequences, with relatively minor sequence variation within these two groups. Thus, two clones representing the two divergent sequence types were selected for additional sequence walking upstream and downstream of the *lax-barrenstalk1* orthogene. Both clones contain 636-bp ORFs homologous to the single-exon 648-bp *O. lax* (AB115668) and the single-exon 660-bp *Zea barrenstalk1* (AY683001) orthogenes. Moreover, deduced amino-acid (aa) sequences from the two divergent *Leymus* BAC clones, FJ373309 and FJ373310, show greatest homology to the *O. lax* (AB115668) and *Zea barrenstalk1* (AY683001) orthogenes when searched against the GenBank nucleotide collection. The 240-place alignment of predicted proteins from the putative *Leymus* (211 aa), *Oryza* (223 aa), *Zea* (219 aa), and *Sorghum* (232 aa) *lax-barrenstalk1* genes shows 111 conserved residues, 21 functionally conserved residue substitutions, and 21 functionally semi-conserved residue substitutions with complete conservation of the putative bHLH DNA-binding domain [27,28] (Fig. 2). Relationships based on the percent divergence in the deduced *lax-barrenstalk1* proteins (Table 1, Fig. 2) show two groups with high bootstrap confidence levels: (1) *Zea* and *Sorghum* with about 12% difference, and (2) *Leymus* LG3a and *Leymus* LG3b with about 5% divergence. The *O. lax-barrenstalk1* protein is more similar to the *Leymus* based on percent amino-acid similarity (Fig. 2), but not supported by statistically meaningful bootstrap confidence levels.

3.4. Linkage mapping and alignment to the O. lax-barrenstalk1 orthogene

Gene specific PCR primers designed from non-coding sequences of two divergent *Leymus* BAC clones (FJ373309 and FJ373310),

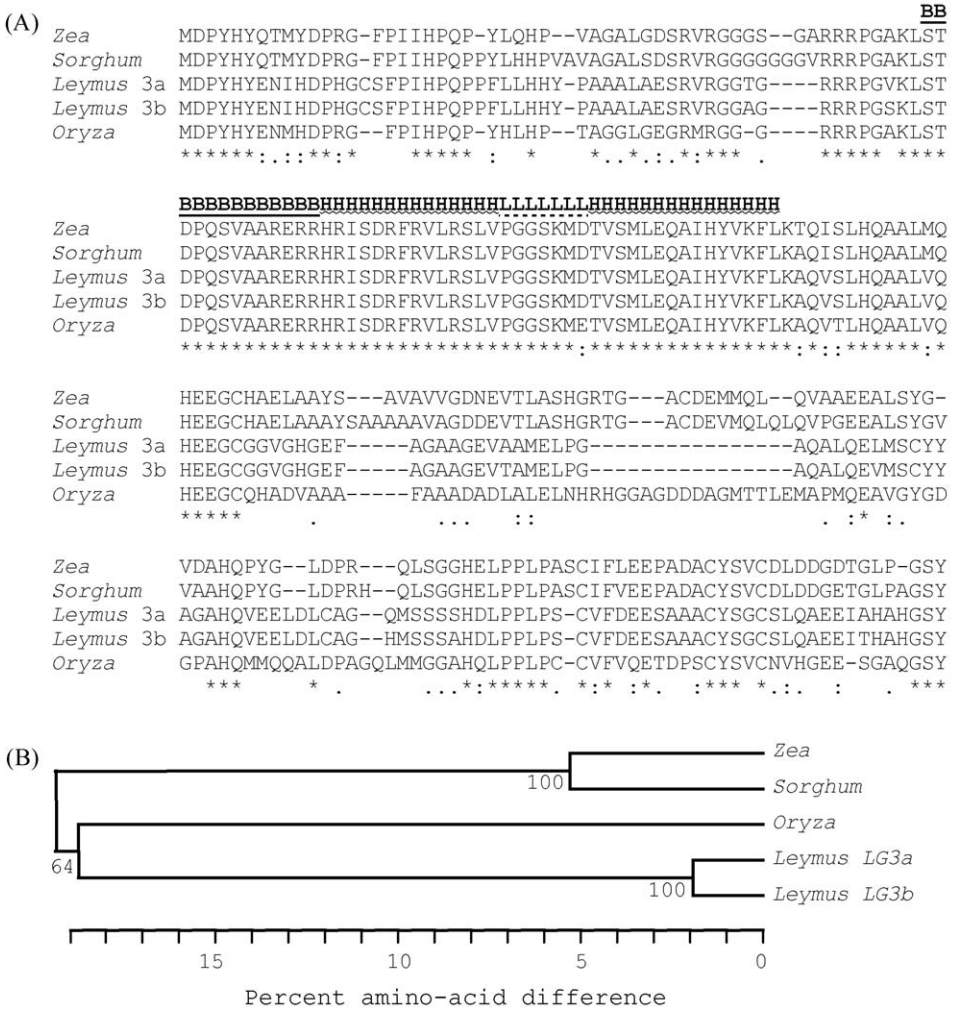

Fig. 2. Comparison of deduced amino-acid residues from the *Leymus* LG3a (FJ373309), *Leymus* LG3b (FJ373310), *Zea barrenstalk1* (AY683001), *Oryza lax* (AB115668), and *Sorghum* (XM_002458669) orthogenes. (A) Multiple-sequence alignment of the predicted *lax-barrrenstalk1* transcription factor proteins with conserved amino-acid residues, conserved substitutions, and semi-conserved substitutions are indicated by "*", ":", and "." symbols, respectively. The basic helix-loop-helix (bHLH) domain regions are indicated in bold as **B** (basic), **H** (helix), and **L** (loop). (B) Dendogram of UPGMA cluster analysis based on percent similarity among deduced amino-acid sequences of the *lax-barrrenstalk1* orthogenes, with 50% majority-rule bootstrap confidence levels.

Table 1
Percent nucleotide change (lower diagonal) or percent similarity (upper diagonal) among the maize (*Zea mays*) *barrenstalk1* protein and deduced amino-acid sequences of the rice *lax* (*Oryza sativa*), sorghum (*Sorghum bicolor*), and wildrye (*Leymus cinereus* × *triticoides*) orthogenes.

	Zea	*Sorghum*	*Oryza*	*Leymus* LG3a	*Leymus* LG3b
Zea	0	89	58	57	57
Sorghum	11	0	62	62	62
Oryza	42	38	0	61	61
Leymus LG3a	43	38	39	0	96
Leymus LG3b	43	38	39	4	0

upstream of the putative *lax-barrenstalk1* orthogene sequences, detected polymorphisms in the *Leymus* TTC1 and TTC2 mapping populations. The LG3a.57-407F and LG3a.57-034R primer pair, designed from non-coding regions from one of the two divergent *Leymus* BAC clones (FJ373309) containing putative *lax-barrenstalk1* orthogene sequences, amplified a 367-bp band that mapped to the long arm of *Leymus* homoeologous group LG3a (Fig. 3). The LG3b.57-640F and LG3b.57-035R primer pair, designed from non-coding regions of other *Leymus* BAC clone (FJ373310) containing putative *lax-barrenstalk1* orthogene sequences, amplified a 590-bp band that mapped to the long arm of *Leymus* homoeologous group LG3b (Fig. 3). The putative *lax-barrenstalk1* orthogenes are located on the long arms of *Leymus* homoeolgous LG3a and LG3a are both most homologous to the same *lax-barrenstalk1* orthogene on the *Oryza* P0446G04 BAC clone on *Oryza* chromosome 1 (Fig. 3), when searched against the GenBank nucleotide collection or *Oryza* WGS (as described in Sections 3.2 and 3.3 above). Moreover, the putative *Leymus* and *O. lax-barrenstalk1* orthogenes-*barrenstalk1* orthogenes and other conserved gene sequences are syntenous and show co-linear map orders on the long arms of *Leymus* homoeologous group 3 and *Oryza* chromosome 1 (Fig. 3). These map data and sequence homology searches provide compelling evidence that the *Leymus* LG3a FJ373309 and LG3b FJ373310 loci contain genes that are orthologous to the *O. lax-barrenstalk1* orthogenes (AB115668) and *Zea barrenstalk1* (AY683001) genes.

3.5. Comparisons of Leymus, Oryza, Sorghum, and Zea lax-barrenstalk1 orthogenes

Self-comparisons of genomic DNA sequences, using dot-plot and percent identity plots (Fig. 4) show dispersed, tandem, and inverted repeat elements in *Sorghum* and *Oryza*, some of which are conserved between these two divergent genera. Conversely, self-comparisons of genomic DNA sequences evidently show smaller and more localized repeat elements in *Zea* and *Leymus* LG3b and only one relatively small repeat structure in the *Leymus* LG3a sequences.

Comparisons of the *lax-barrenstalk1*orthogene and flanking genomic regions between the *Leymus* LG3a (FJ373309) and *Leymus* LG3b (FJ373310) using dot-plot and percent-identify plots (Fig. 4) elucidate patterns of sequence divergence, insertion and deletion, and unique sequences that differentiate these homoeologous loci. In particular, the PIP analysis detected 3835 bp of sequence showing greater than 50% conservation between the 7233-bp *Leymus* LG3a FJ373309 and 7683-bp LG3b FJ373310 *Leymus* BAC sequences (Fig. 4). Thus, about 53% and 48% of the homoelogous *Leymus* LG3a FJ373309 and LG3b FJ373310 BAC clones are conserved relative to each other.

Comparisons of the *lax-barrenstalk1*orthogene and flanking genomic regions among *Leymus* LG3a (FJ373309), *Leymus* LG3b (FJ373310), *Zea* (AY683001), *Oryza* (AB115668), and *Sorghum* chromosome 2 revealed two conserved regions (CR2, and CR3) and a third conserved region (CR3) that was also present in *Leymus* LG3a, *Oryza*, and *Sorghum* sequences (Fig. 4). The largest conserved region (CR2) is the *lax-barrenstalk1*orthogene itself, which includes 649 bp of *O. lax-barrenstalk1* orthogenes-*barrenstalk1* coding sequence, 633 bp of coding sequence in both of the *Leymus* LG3a FJ373309 and *Leymus* LG3b FJ373310 BAC clones, 657 bp of coding sequence in *Zea*, and 696 bp of coding sequence in *Sorghum* in addition to other 5′ and 3′ homologous sequences adjacent to these coding sequences. Another conserved region (CR1) located upstream of the *lax-barrenstalk1* orthogene (CR2) was present in the *Leymus* LG3a, *Oryza*, and *Sorghum* genomic DNA sequences (Fig. 4). A third conserved region (CR3) is located downstream of the *lax-barrenstalk1* orthogene (CR2) in the *Leymus* LG3a, *Leymus* LG3b, *Zea*, and *Sorghum* genomic DNA sequences and inverted upstream of the *lax-barrenstalk1* orthogene (CR2) in *Oryza* (Fig. 4). Thus, the *lax-barrenstalk1* orthogene (CR2) and CR3 are present in all sequences, but CR1 is absent in the *Leymus* LG3a and *Zea*.

3.6. Nucleotide polymorphism in the Leymus lax-barrentalk1 orthogenes

The GSP.057F and GSP.210R primers were used to amplify and resequence coding regions of the *Leymus lax-barrentalk1* orthogene from the heterogeneous *L. triticoides* Acc641 and *L. cinereus* Acc636 parental accessions and compare these sequences to mapped (Fig. 5) BAC DNA sequences from the *L. triticoides* Acc641 × *L. cinereus* Acc636 TC2 hybrid. The *Leymus* LG3a FJ373309 and LG3b FJ373310 *lax-barrentalk1* coding regions showed 16 single-nucleotide polymorphisms (SNPs) resulting in seven amino-acid substitutions (Fig. 5). The GSP.057F and GSP.210R primers flank nine of the 16 SNPs that distinguish LG3a FJ373309 and LG3b FJ373310 *lax-barrentalk1* coding regions (Fig. 5). In addition to sequences that were identical or nearly identical to the LG3a FJ373309 or LG3b FJ373310 BAC DNA sequences, we detected at least two sequence variants (FJ373311 and FJ373312) from *L. triticoides* (CWR) using the GSP.057F and GSP.210R PCR primers (Fig. 5). Compared over the nine SNPs that distinguish LG3a FJ373309 and LG3b FJ373310 loci, both of the *L. triticoides* FJ373311 and FJ373312 sequences were more similar to the LG3a FJ373309 BAC sequence (Fig. 5).

The *L. triticoides* FJ373312 sequence displayed a 3-bp deletion, resulting in the loss of a leucine residue, and six other silent SNP mutations that were unique to this amplicon (Fig. 5). Otherwise, the FJ373312 sequence was identical to LG3a FJ373309 sequence, including all nine SNPs that distinguish the LG3a and LG3b loci (Fig. 5). Thus, we deduce that the *L. triticoides* FJ373312 sequence is allelic to the LG3a FJ373309 locus (Fig. 5).

The *L. triticoides* FJ373311 sequence contains a 17-bp deletion, relative to other *Leymus lax-barrentalk1* coding sequences (Fig. 5), which creates codon reading frameshift with a premature stop codon (TGA) located between nucleotides 165 and 167 (Fig. 5). The *L. triticoides* FJ373311 sequence variant also contains a 8-bp insertion and two other SNP mutations, relative to the other *Leymus lax-barrentalk1* coding sequences (Fig. 5). The FJ373311 sequence matched the LG3a FJ373309 sequence over six of the nine SNPs and completely lack two of the nine SNPs as a result of the 17-bp deletion (Fig. 5). However, FJ373311 sequence also shared one SNP allele with the LG3b FJ373310 sequence (Fig. 5). Nevertheless, we deduce that the FJ373311 amplicon is a psuedogene allele the LG3a FJ373309 locus.

The combination of 17-bp deletion and 8-bp insertion of the *L. triticoides* FJ373311 sequence variant (Fig. 5) was visible on agarose by PCR amplification of genomic DNA from the T-tester *L. triticoides* parent and segregates in an approximate 1:1 ratio in both TTC1 and TTC2 populations (data not shown), using the GSP.057f and GSP.474r primer pair (Table S1). However, this *lax-barrentalk1* size variant was not detected using RT-PCR and cDNA in the TTC1 and TTC2 progeny.

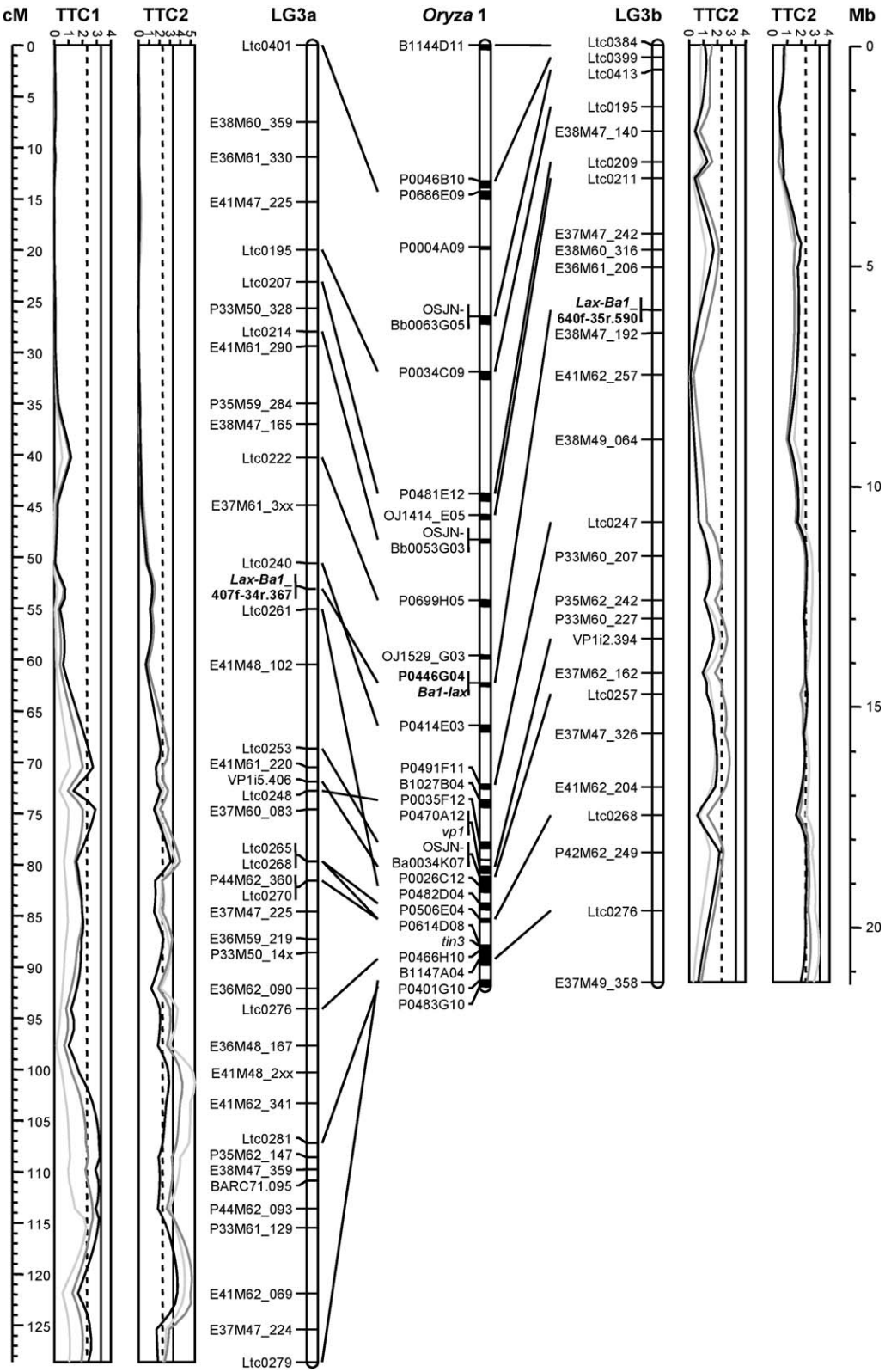

Fig. 3. Comparative mapping of *lax-barrenstalk1* orthogenes and *Leymus* rhizome QTLs based on molecular genetic recombination maps for the long arms of *Leymus* LG3a and LG3b; LOD scans of the *Leymus* TTC1 and TTC2 rhizome spreading QTLs; and physical map of corresponding gene sequences on the long arm of *Oryza* chromosome 1. The approximate 5% chromosome-wide and genome-wide LOD thresholds are shown as *dashed lines* (LOD = 2.3) and *solid lines* (LOD = 3.3). The recombination distances (cm) for the LG3a and LG3b consensus maps from of the *Leymus* TTC1 and TTC2 mapping populations is shown on the *left side* of figure, whereas the physical location and coverage of *Oryza* chromosome 1 BAC clones is shown (in base pair units) on the *right side* of figure.

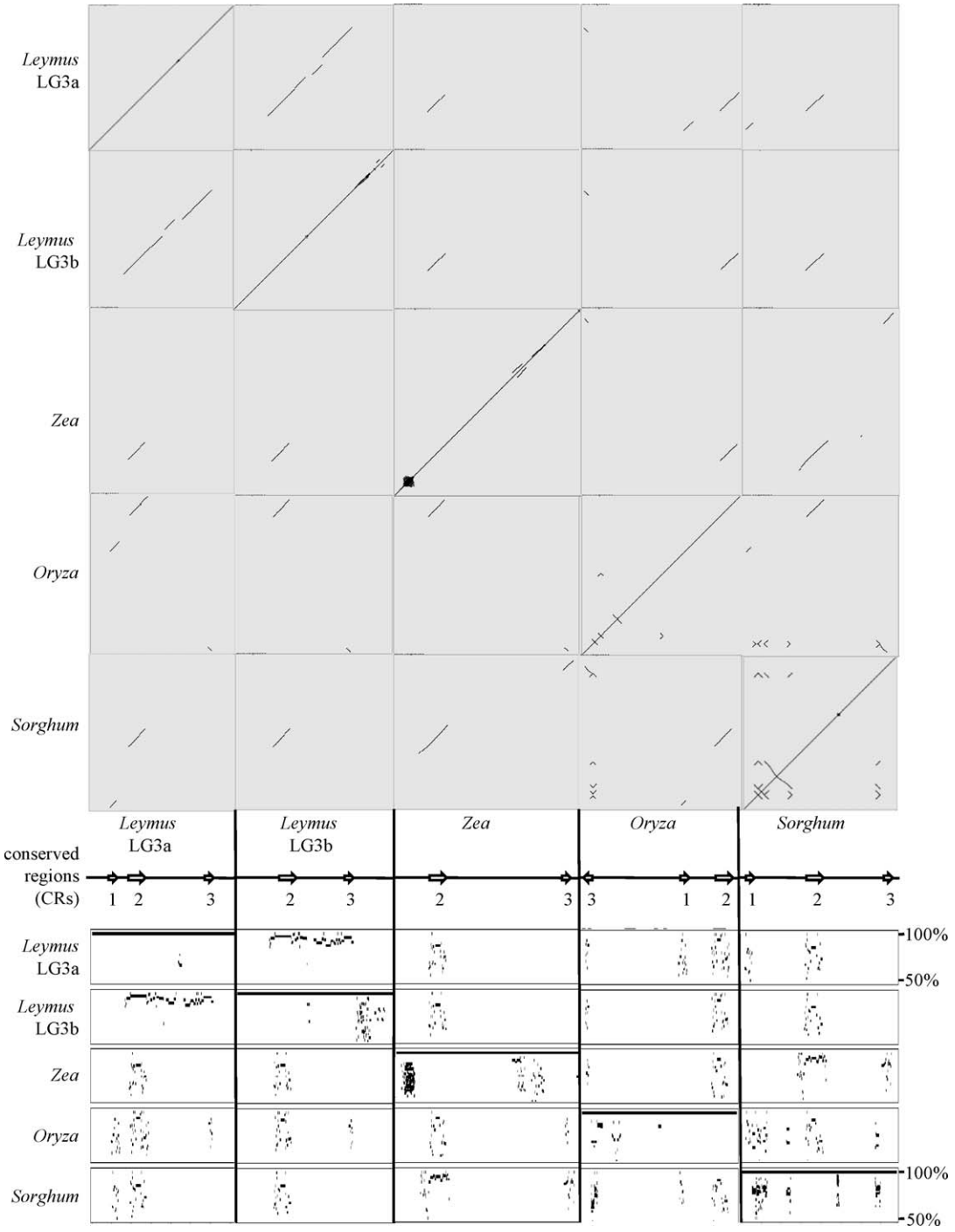

Fig. 4. Pairwise comparisons of dot-plot sequence alignments and percent identity plots (PIPs) among *Leymus* LG3a (FJ373309), *Leymus* LG3b (FJ373310), *Zea barrenstalk1* (AY683001), *Oryza lax* (AB115668), and *Sorghum* chromosome 3 genomic sequences containing *lax-barrenstalk1* orthogenes. The inferred orientation of three highly conserved regions (CRs) including coding sequences of the *lax-barrenstalk1* orthogene (CR2) are shown.

4. Discussion

4.1. Identification of lax-barrenstalk1 orthogene in cool-season grasses

Discovery of the *lax-barrenstalk1* orthogene in *Leymus* is somewhat surprising considering that this gene was not previously found in the EST libraries of wheat, barley, or other cool-season cereals and grasses. However, the absence of this gene in EST libraries of wheat, barley, and other cool-season cereals may not be totally unexpected since expression of transcription factors is low and also because there has been relatively little attention given to the axillary meristems of these species. Most of the wheat and barley EST libraries were constructed using stems, leaves, flowers, seeds, and roots. Rhizomes are common and important features of perennial grasses, but virtually absent or sparse in cereals and other annual grasses.

With an average insert size of 150.5 kb, the 405,888 *Leymus* BAC clones developed in this project represent approximately 6.1× haploid genomes of tetraploid *Leymus*. Hybridization of an overgo

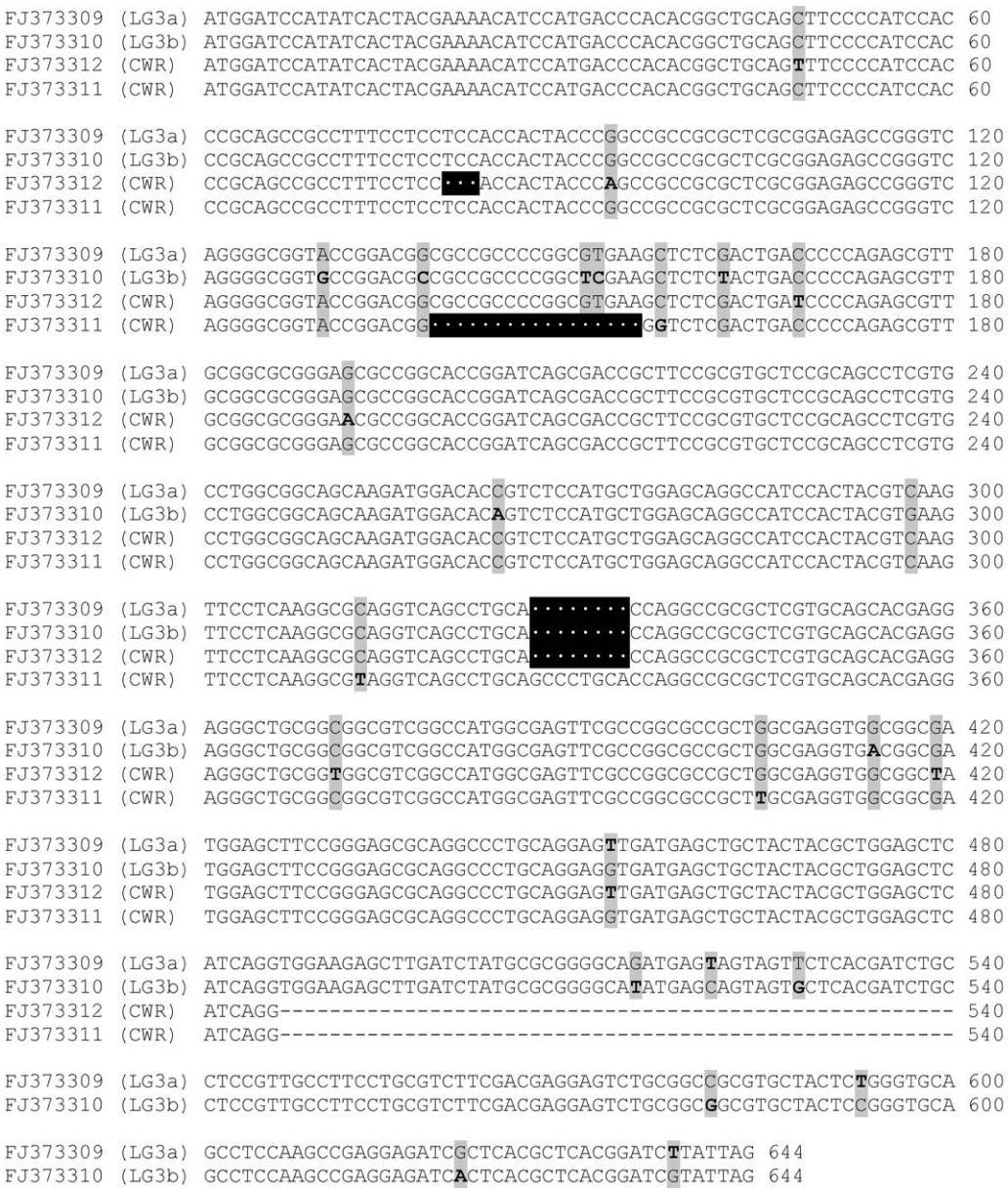

Fig. 5. Nucleotide sequence and length variation in *Leymus lax-barrenstalk1* orthogene sequences from LG3a and LG3b BAC clones and creeping wildrye (CWR) resequencing.

probe, designed from the single-copy l*ax-barrenstalk1* orthogene, to twelve *Leymus* BAC colonies and identification of orthologous *lax-barrenstalk1* sequences in at least seven of these clones confirm effective genome representation of this library.

Evidence that the *lax-barrenstalk1* orthogene [27,28] is present in cool-season grasses based on sequence homology and mapping data. In particular, the *Leymus* FJ373309 and FJ373310 BAC clones contain nucleotide and deduced amino-acid sequences that show greatest homology to the *O. lax-barrenstalk1* orthogenes panicle [27] and *Zea barrenstalk1* [28] orthogenes, even when searched against the entire NCBI GenBank nucleotide collection, the NCBI *Oryza* WGS, or the *Sorghum* WGS. Moreover, the putative bHLH DNA-binding domain of the *lax-barrenstalk1* orthogene [27,28] is highly conserved among the five grass gene sequences (Fig. 2), which is interesting given that these proteins lack the glutamic acid residue at position nine in the basic region that was demonstrated in non-plant bHLH proteins to be necessary for DNA binding to the canonical E-box site [28,59–61]. Thus, the grass *lax-barrenstalk1* orthogene belongs to a subgroup of proteins that share an atypical bHLH domain, which was suggested to bind a different sequence motiff [28,59–61]. The orthology of *Leymus* and *O. lax-barrenstalk1* orthogenes-*barrenstalk1* sequences is also evident by synteny and co-linearity of *Leymus* LG3a, *Leymus* LG3b, and *Oryza* chromosome 1 DNA sequences including the putative *lax-barrenstalk1* ortholoci (Fig. 5). Moreover, amplification of the *lax-barrenstalk1* transcripts (Sections 3.2 and 3.6 above) from cDNA prepared from subterranean rhizome and tiller buds of *Leymus* [51] suggest that this gene be involved in the formation of axillary meristems in *Leymus* wildryes, as it is in *Oryza* [27] and *Zea* [28]. Taken together, these findings provide convincing evidence that the *Leymus* FJ373309 and FJ373310 BAC clones contain functional *lax-barrenstalk1* orthogenes that may be involved in the initiation of rhizome and tiller bud meristems, even if it is not directly responsible for the *Leymus* homoeologous group 3 QTLs controlling growth habit differences between caespitose *L. cinereus* and rhizomatous *L. triticoides*.

4.2. Amino-acid sequence divergence among the Poaceae lax-barrenstalk1 orthogenes and identification of upstream and downstream conserved DNA sequences

Cool-season Pooideae grasses including the Triticeae cereals wheat, barley, and rye and perennial Triticeae grasses such as the *Leymus* wildryes diverged from the Oryzoideae (including genus *Oryza*) and Panicoideae (including the genera *Zea* and *Sorghum*) 47.2–62.3 and 55–72.2 million years ago (MYA), respectively [62–64]. Thus, the general relationships among *Leymus*, *Oryza*, *Sorghum*, and Zea l*ax-barrenstalk1* orthogenes (Table 1, Fig. 2) is very much consistent with known phylogenetic relationships among these Poaceae species. Within Triticeae, *Hordeum* originated 10.1–11 MYA [65,66] followed by *Secale* ~7 MYA [65], and *Triticum–Aegilops* complex 2.5–4.5 MYA [67]. Of the diploid progenitors of wheat, the B genome diverged from the A–D lineage 2.5–6 MYA [68] and A from D between 2.7 and less than 0.5 MYA [65,66]. Thus, the observed divergence between the homoeologous *Leymus* LG3a and *Leymus* LG3b *lax-barrenstalk1* orthogenes appears to be within the range of divergence observed among various Triticeae genomes and subgenomes, based on approximate comparisons of relative amino-acid sequence differences among other Poaceae *lax-barrenstalk1* orthogenes analyzed in this study (Fig. 2).

Only one of the three conserved regions among *Leymus*, *Oryza*, *Sorghum*, and *Zea* genomic DNA sequences, the CR2 l*ax-barrenstalk1* orthogene sequence (Fig. 4), has been annotated in the NCBI GenBank. Likewise, the only sequence of the *Leymus* LG3a FJ373309 and LG3b FJ373310 BAC clones that shows conservation with deduced translated sequences of these databases is the CR3 l*ax-barrenstalk1* coding region. Although neither the CR2 nor CR3 regions have been annotated in *Oryza, Sorghum*, or *Zea*, it is possible that one or both of these sequences are regulatory sequences of the l*ax-barrenstalk1* orthogene. Conservation of linear order and orientation of CR1, CR2, and CR3 between *Leymus* LG3a and *Sorghum* genomic sequences may represent the ancestral configuration of grasses.

Sequence alignments of the *Leymus* LG3a FJ373309 and LG3b FJ373310 *lax-barrenstalk1* genomic DNA regions provides the first glimpse of DNA sequence divergence and rearrangements between homoeologous loci of tetraploid *Leymus*. One of the three conserved regions (CR1) between the *Leymus* LG3a FJ373309 BAC clone and *Oryza* is not even present in the *Leymus* LG3b FJ373310 clone (Fig. 4). Nearly half of the *Leymus* LG3a FJ373309 and LG3b FJ373310 DNA sequence alignments show less than 50% (no significant) homology. Regions of homology between the *Leymus* LG3a FJ373309 and LG3b FJ373310 clones are broken by two regions of sequence divergence and show somewhat different relative positions (Fig. 4). These data demonstrate a molecular basis for the lack of multivalent formation during chromosome pairing and the display of consequent disomic inheritance observed in *Leymus*, which are consistent with the allotetraploid genome interpretation for *Leymus* [15,38–40,42].

Differences between genomic DNA sequences of the *Leymus* LG3a FJ373309 and LG3b FJ373310 BAC clones (Fig. 4) are similar to differences observed between homoeologous loci of wheat. Comparisons of genomic DNA between subgenomes of hexaploid wheat are still limited, but often show gene deletions, localized gene rearrangements, and numerous other insertion and deletion events driven by changes in the number and distribution of retrotransposons [69,70]. Although some local rearrangements between *Leymus* and *Oryza* were detectable (Fig. 4), larger expanses of genomic DNA may be needed to fully elucidate homology and divergence between subgenomes of allotetraploid *Leymus*.

4.3. Nucleotide polymorphism and possible QTL associations involving the Leymus lax-barrentalk1 orthogene

The null *lax-barrenstalk1* allele *L. triticoides* is somewhat surprising considering that *L. triticoides* generally displays more tillers and rhizome branches compared to *L. cinereus*. Loss of *lax-barrenstalk1* gene function would be expected to abbreviate initiation of lateral branch meristems based on phenotypic effects observed in maize [28] and rice [27]. Thus, it seems unlikely that this null allele provides explains growth habit differences between *L. cinereus* and *L. triticoides*. Moreover, the LG3a *lax-barrenstalk1* locus was not aligned to *L. triticoides* × (*L. triticoides* × *L. cinereus*) TTC1 and TTC2 rhizome QTLs located on the more distal region of the LG3a long arm (Fig. 3) [4]. However, the tetraploid hybrids (TC1 and TC2) and the *L. triticoides* tester parent (T-Tester) used to construct the TTC1 and TTC2 QTL mapping populations [4] each contain up to 4 possible alleles. Even though the TTC1 and TTC2 molecular maps were constructed using only markers present in the hybrids (TC1 or TC2) and absent in the tester genotype, it is possible to have up to eight different alleles segregating in the TTC1 and TTC2 populations. Thus, we are not certain as to whether we were able to directly test effects of the null mutation because the markers we used for mapping did not flank this mutation *per se*. Although the *lax-barrenstalk1* orthogene may not be responsible for differences in the circumference of rhizome spreading between *L. triticoides* and *L. cinereus* [4], it is may still be involved in the formation of axillary meristems including tiller and/or rhizome buds of these and other cool-season perennial grasses.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2009.07.006.

References

[1] L.L. Qi, S.L. Wang, P.D. Chen, D.J. Liu, B. Friebe, B.S. Gill, Molecular cytogenetic analysis of *Leymus racemosus* chromosomes added to wheat, Theor. Appl. Genet. 95 (1997) 1084–1091.
[2] M. Kishi, T. Yamada, T. Sasakuma, H. Tsujimoto, Production of wheat-*Leymus racemosus* chromosome addition lines, Theor. Appl. Genet. 109 (2004) 255–260.
[3] G.V. Subbarao, B. Tomohiro, K. Masahiro, I. Osamu, H. Samejima, H.Y. Wang, S.J. Pearse, S. Gopalakrishnan, K. Nakahara, A.K.M. Zakir Hossain, H. Tsujimoto, W.L. Berry, Can biological nitrification inhibition (BNI) genes from perennial *Leymus racemosus* (Triticeae) combat nitrification in wheat farming? Plant Soil 299 (2007) 55–64.
[4] S.R. Larson, X.-L. Wu, T.A. Jones, K.B. Jensen, N.J. Chatterton, B.L. Waldron, J.G. Robins, B.S. Bushman, A.J. Palazzo, Comparative mapping of growth habit, plant height, and flowering QTLs in two interspecific families of *Leymus*, Crop Sci. 46 (2006) 2526–2539.
[5] A.L. Lesperance, J.A. Young, R.E. Eckert, R.A. Evans, Great basin wildrye, Rangeman's J. 5 (1978) 125–127.
[6] R.A. Evans, J.A. Young, 'Magnar' basin wildrye—germination in relation to temperature, J. Range Manage. 36 (1983) 395–398.
[7] J.A. Young, R.A. Evans, Germination of great basin wildrye seeds collected from native stands, Agron. J. 73 (1981) 917–920.
[8] R.F. Miller, F.A. Branson, I.S. McQueen, C.T. Snyder, Water relations in soils as related to plant communities in Ruby, Valley, Nevada, J. Range Manage. 35 (1982) 462–468.
[9] B.A. Roundy, Response of basin wildrye and tall wheatgrass seedlings to salination, Agron. J. 75 (1983) 67–71.
[10] B.A. Roundy, J.A. Young, R.A. Evans, Germination of basin wildrye and tall wheatgrass in relation to osmotic and matric potential, Agron. J. 77 (1985) 129–135.
[11] H. Suyama, S.E. Benes, P.H. Robinson, G. Getachew, S.R. Gratten, C.M. Grieve, Biomass yield and nutritional quality of forage species under long-term irrigation with saline-sodic drainage water: field evaluation, Anim. Feed Sci. Technol. 135 (2007) 329–345.
[12] H. Suyama, S.E. Benes, P.H. Robinson, S.R. Gratten, C.M. Grieve, G. Getachew, Forage yield and quality under irrigation with saline-sodic drainage water: greenhouse evaluation, Agric. Water Mange. 88 (2007) 159–172.
[13] Z.-M. Hu, S.R. Larson, T.A. Jones, R.R.-C. Wang, Genetic relationships among soluble carbohydrates, anthocyanins, and growth characteristics in *Leymus* (Gramineae) detected with molecular markers, Acta Bot. Sin. 44 (2002) 1173–1181.

[14] S.R. Larson, K.B. Jensen, J.G. Robins, T.A. Jones, Identification of species, genes, and traits appropriate for low-input biomass production in western North America, in: Abstracts of the GSA, SSSA-ASA-CSSA, GCAGS Joint Annual Meeting, Houston, TX, 2008.
[15] X.-L. Wu, S.R. Larson, Z.-M. Hu, A.J. Palazzo, T.A. Jones, R.R.-C. Wang, K.B. Jensen, N.J. Chatterton, Molecular genetic linkage maps for allotetraploid *Leymus* (Triticeae), Genome 46 (2003) 627–646.
[16] P. Kaur, S.R. Larson, B.S. Bushman, R.R.-C. Wang, I.W. Mott, D. Hole, J. Thimmapuram, G. Gong, L. Liu, Genes controlling plant growth habit in *Leymus* (Triticeae): *Zea barren stalk1* (*ba1*), *Oryza lax* panicle, and wheat *tiller inhibition* (*tin3*) genes as possible candidates, Funct. Integr. Genomics 8 (2008) 375–386.
[17] B.S. Bushman, S.R. Larson, I.W. Mott, P.F. Cliften, R.R.-C. Wang, N.J. Chatterton, A.G. Hernandez, S. Ali, R.W. Kim, J. Thimmapuram, G. Gon, L. Liu, M.A. Mikel, Development and annotation of perennial Triticeae ESTs and SSR markers, Genome 51 (2008) 779–788.
[18] S.R. Larson, H.F. Mayland, Comparative mapping of fiber, protein, and mineral content QTLs in two interspecific *Leymus* wildrye full-sib families, Mol. Breed. 20 (2007) 331–347.
[19] S.R. Larson, E.A. Kellogg, Genetic dissection of seed production traits and identification of a major-effect seed retention QTL in hybrid *Leymus* (Triticeae) wildryes, Crop Sci. 49 (2009) 29–40.
[20] M. Kishii, T. Yamada, T. Sasakuma, H. Tsujimoto, Production of wheat-*Leymus racemosus* chromosome addition lines, Theor. Appl. Genet. 109 (2004) 255–260.
[21] P. Chen, W. Liu, J. Yuan, X. Wang, B. Zhou, S. Wang, S. Zhang, Y. Feng, B. Yang, G. Liu, D. Liu, L. Qi, P. Zhang, B. Freibe, B.S. Gill, Development and characterization of wheat-*Leymus racemosus* translocation lines with resistance to *Fusarium* Head Blight, Theor. Appl. Genet. 111 (2005) 941–948.
[22] R.E. Oliver, X. Cai, S.S. Xu, X. Chen, R.W. Stack, Wheat-alien species derivatives: a novel source of resistance to *Fusarium* head blight in wheat, Crop Sci. 45 (2005) 1353–1360.
[23] H.-B. Zhang, C.C. Wu, BACs as tools for genome sequencing, Plant Physiol. Biochem. 39 (2001) 195–209.
[24] I.S. Donnison, D.M. O'Sulliban, A. Thomas, P. Canter, B. Moore, I. Armstead, H. Thomas, K.J. Edwards, I.P. King, Construction of a *Festuca pratensis* BAC library for map-based cloning in *Festulolium* substitution lines, Theor. Appl. Genet. 110 (2005) 846–851.
[25] K. Farrar, T. Asp, T. Lűbberstedt, M. Xu, A.M. Thomas, C. Christiansen, M.O. Humphreys, I.S. Donnison, Construction of two *Lolium perenne* BAC libraries and identification of BACs containing candidate genes for disease resistance and forage quality, Mol. Breeding 19 (2007) 15–23.
[26] A.H. Paterson, K.F. Schertz, Y.-R. Lin, S.-C. Liu, Y.-L. Chang, The weediness of wild plants: molecular analysis of genes influencing dispersal and persistance of johnsongrass, *Sorghum halepense* (L.) Pers., Proc. Natl. Acad. Sci. USA 92 (1995) 6127–6131.
[27] K. Komatsu, M. Maekawa, S. Ujiie, Y. Satake, I. Furutani, H. Okamoto, K. Shimamoto, J. Kyozuka, LAX and SPA: major regulators of shoot branching in *Oryza*, Proc. Natl. Acad. Sci. USA 100 (2003) 11765–11770.
[28] A. Gallovotti, Q. Zhao, J. Kyozuka, R.B. Meeley, M.K. Ritter, J.F. Doebley, M.E. Pè, R.J. Schmidt, The role of *barren stalk1* in the architecture of *Zea*, Nature 432 (2004) 630–635.
[29] K.M. Devos, M.D. Gale, Genome relationships: the grass model in current research, Plant Cell 12 (2000) 637–646.
[30] M. La Rota, M.E. Sorrels, Comparative DNA sequence analysis of mapped wheat ESTs reveals the complexity of genome relationships between *Oryza* and wheat, Funct. Integr. Genomics 4 (2004) 34–46.
[31] X. Draye, Y.-R. Lin, X.-Y. Qian, J.E. Bowers, G.B. Burow, P.L. Morrell, D.G. Peterson, G.G. Presting, S.-X. Ren, R.A. Wing, A.H. Paterson, Toward integration of comparative genetic, physical, diversity, and cytomolecular maps for grasses and grains, using the sorghum genome as a foundation, Plant Physiol. 125 (2001) 1325–1341.
[32] F.A. Feltus, G.E. Hart, K.F. Schertz, A.M. Casa, S. Kresovich, S. Abraham, P.E. Klein, P.J. Brown, A.H. Paterson, Alignment of genetic maps and QTLs between inter- and intra-specific sorghum populations, Theor. Appl. Genet. 112 (2006) 1295–1305.
[33] B.L. Hass-Jacobus, M. Futrell-Griggs, B. Abernathy, R. Westerman, J.-L. Goicoechea, J. Stein, P. Klein, B. Hurwitz, B. Zhou, F. Rakhshan, A. Sanyal, N. Gill, J.-Y. Lin, J.G. Walling, M.Z. Luo, J. Siva, S. Ammiraju, D. Kudrna, H.R. Kim, D. Ware, R.A. Wing, P.S. Miguel, S.A. Jackson, Integration of hybridization-based markers (overgos) into physical maps for comparative and evolutionary explorations in the genus *Oryza* and in *Sorghum*, BMC Genomics 7 (2006) 199.
[34] A.H. Paterson, J.E. Bowers, R. Bruggmann, I. Dubchak, J. Grimwood, H. Gundlach, G. Haberer, U. Hellsten, T. Mitros, A. Poliakov, J. Schmutz, M. Spannagl, H. Tang, X. Wang, T. Wicker, A.K. Bharti, J. Chapman, F.A. Feltus, U. Gowik, I.V. Grigoriev, E. Lyons, C.A. Maher, M. Martis, A. Narechania, R.P. Otillar, B.W. Penning, A.A. Salamov, Y. Wang, L. Zhang, N.C. Carpita, M. Freeling, A.R. Gingle, C.T. Hash, B. Keller, P. Klein, S. Kresovich, M.C. McCann, R. Ming, D.G. Peterson, M.-u. -Rahman, D. Ware, P. Westhoff, K.F.X. mayer, J. Messing, D.S. Rokshar, The *Sorghum bicolor* genome and the diversification of grasses, Nature 457 (2009) 551–556.
[35] K.P. Vogel, A. Arumuganathan, K.B. Jensen, Nuclear DNA content of perennial grasses of the Triticeae, Crop Sci. 39 (1999) 661–667.
[36] J. Yu, S. Hu, J. Wang, G.K.-S. Wong, S. Li, B. Liu, Y. Deng, L. Dai, Y. Zhou, X. Zhang, M. Cao, J. Liu, J. Sun, J. Tang, Y. Chen, X. Huang, W. Lin, C. Ye, W. Tong, L. Cong, J. Geng, Y. Han, L. Li, W. Li, G. Hu, X. Huang, W. Li, J. Li, Z. Liu, L. Li, J. Liu, Q. Qi, J. Liu, L. Li, T. Li, X. Wang, H. Lu, T. Wu, M. Zhu, P. Ni, H. Han, W. Dong, X. Ren, X. Feng, P. Cui, X. Li, H. Wang, X. Xu, W. Zhai, Z. Xu, J. Zhang, S. He, J. Zhang, J. Xu, K. Zhang, X. Zheng, J. Dong, W. Zeng, L. Tao, J. Ye, J. Tan, X. Ren, X. Chen, J. He, D. Liu, W. Tian, C. Tian, H. Xia, Q. Bao, G. Li, H. Gao, T. Cao, J. Wang, W. Zhao, P. Li, W. Chen, X. Wang, Y. Zhang, J. Hu, J. Wang, S. Liu, J. Yang, G. Zhang, Y. Xiong, Z. Li, L. Mao, C. Zhou, Z. Zhu, R. Chen, B. Hao, W. Zheng, S. Chen, W. Guo, G. Li, S. Liu, M. Tao, J. Wang, L. Zhu, L. Yuan, H. Yang, A draft sequence of the *Oryza* genome (*Oryza sativa* L. ssp. *indica*), Science 296 (2002) 79–92.
[37] S.A. Goff, D. Ricke, T.-H. Lan, G. Presting, R. Wang, M. Dunn, J. Glazebrook, A. Sessions, P. Oeller, H. Varma, D. Hadley, D. Hutchison, C. Martin, F. Katagiri, B.M. Lange, T. Moughamer, Y. Xia, P. Budworth, J. Zhong, T. Miguel, U. Paszkowski, S. Zhang, M. Colbert, W. Sun, L. Chen, B. Cooper, S. Park, T.C. Wood, L. Mao, P. Quail, R. Wing, R. Dean, Y. Yu, A. Zharkikh, R. Shen, S. Sahasrabudhe, A. Thomas, R. Cannings, A. Gutin, D. Pruss, J. Reid, S. Tavtigian, J. Mitchell, G. Eldredge, T. Scholl, R.M. Miller, S. Bhatnagar, N. Adey, T. Rubano, N. Tusneem, R. Robinson, J. Feldhaus, T. Macalma, A. Oliphant, S. Briggs, A draft sequence of the *Oryza* genome (*Oryza sativa* L. ssp. *japonica*), Science 296 (2002) 92–100.
[38] D.R. Dewey, Genome relations among diploid *Elymus junceus* and certain tetraploid and octoploid *Elymus* species, Am. J. Bot. 57 (1970) 633–639.
[39] D.R. Dewey, The genomic system of classification as a guide to intergeneric hybridization with the perennial Triticeae, in: J.P. Gustafson (Ed.), Proceedings of the 16th Stadler Genetics Symposium, Plenum, New York, 1984.
[40] Á. Löve, Conspectus of the Triticeae, Feddes Rep. 95 (1984) 425–521.
[41] H.-B. Zhang, J. Dvorak, The genome origin of tetraploid species of *Leymus* (Poaceae: Triticeae) inferred from variation in repeated nucleotide sequences, Am. J. Bot. 78 (1991) 871–884.
[42] R.R.-C. Wang, K.B. Jensen, Absence of the J genome in *Leymus* species (Poaceae: Triticeae): evidence from DNA hybridization and meiotic pairing, Genome 37 (1994) 231–235.
[43] K. Anamthawat-Jónsson, The *Leymus* N^S-Genome, Czech. J. Genet. Plant Breed. 41 (2005) 13–20.
[44] H.-B. Zhang, Construction and Manipulation of Large-insert Bacterial Clone Libraries—Manual, Texas A&M University, TX, USA, 2000.
[45] J. Feng, B.A. Vick, M.-K. Lee, H.-B. Zhang, C.C. Jan, Construction of BAC and BIBAC libraries from sunflower and identificaiton of linkage group-specific clones by overgo hybridization, Theor. Appl. Genet. 113 (2006) 23–32.
[46] C. Wu, Z. Xu, H.-B. Zhang, DNA Libraries, in: R.A. Meyers (Ed.), 2nd ed., Encyclopedia of Molecular Cell Biology and Molecular Medicine, vol. 3, Wiley-VCH Verlag GmbH, Weinheim, Germany, 2004, pp. 385–425.
[47] C. Ren, Z.Y. Xu, S. Sun, M.-K. Lee, C. Wu, C. Scheuring, T.S. Santos, H.-B. Zhang, Genomic DNA libraries and physical mapping, in: K. Meksem, G. Kahl (Eds.), The Handbook of Plant Genome Mapping: Genetic and Physical Mapping, Wiley-VCH Verlag GmbH, Weinheim, Germany, 2005, pp. 173–213.
[48] L. He, C. Du, Y. Li, C. Scheuring, H.-B. Zhang, Large-insert bacterial clone libraries and their applications, in: Z. Liu (Ed.), Aquaculture Genome Technologies, Blackwell Publishing, Ames, Iowa, USA, 2007, pp. 215–244.
[49] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989.
[50] H.-B. Zhang, S. Choi, S.-S. Woo, Z. Li, R.A. Wing, Construction and characterization of two *Oryza* bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population, Mol. Breed. 2 (1996) 11–24.
[51] P. Kaur, I.W. Mott, S.R. Larson, B.S. Bushman, A.G. Hernandez, W.R. Kim, L. Liu, M.A. Mikel, Gene expression polymorphisms and ESTs associated with gravitropic response of subterranean branch meristems and growth habit in *Leymus* wildryes, Plant Sci. 175 (2008) 330–338.
[52] W.W. Cai, J. Reneker, C.W. Chow, M. Vaishnav, A. Bradley, An anchored framework BAC map of mouse chromosome 11 assembled using multiplex oligonucleotide hybridization, Genomics 54 (1998) 387–397.
[53] C.J. Lawrence, L.C. Harper, M.L. Shaeffer, T.Z. Sen, T.E. Seigfied, D.A. Campbell, MaizeGDB: the *Zea* model organism database for basic, translational, and applied research, Int. J. Plant Genomics (2008) 496957.
[54] S.E. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, Mol. Biol. 215 (1990) 403–410.
[55] S. Schwartz, Z. Zhang, K.A. Frazer, A. Smit, C. Riemer, J. Bouck, R. Gibbs, R. Hardison, W. Miller, PipMaker—a web server for aligning two genomic DNA sequences, Genome Res. 10 (2000) 577–586.
[56] M.A. Larkin, G. Blackshield, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, Clustal W and Clustal X version 2.0, Bioinform. Appl. Note 23 (2007) 2947–2948.
[57] J. Benham, J. Jasieniuk, V. Kanazin, T. Blake, Genographer: a graphical tool for automated AFLP and microsatellite analysis, J. Agric. Genomics 4 (1999), article 4, Available from http://wheat.pw.usda.gov/jag/ (accessed 10 February, 2009).
[58] J.W. Van Ooijen, JoinMap® 4.0, Software for Calculation of Genetic Linkage Maps in Experimental Populations, Kyazma, Wageningen, 2006.
[59] M.E. Massari, C. Murre, Helix-loop-helix proteins: regulators of transcription in eukaryotic organisms, Mol. Cell. Biol. 20 (2000) 429–440.
[60] G.E. Toledo-Ortiz, E. Huq, P.H. Quail, The *Arabidopsis* basic/helix-loop-helix transcription factor family, Plant Cell 15 (2003) 1749–1770.
[61] S.J. Liljegren, A.H.K. Roeder, S.A. Kempin, K. Gremski, L. Ostergaard, S. Guimil, D.K. Reyes, M.F. Yanofsky, Control of fruit patterning in *Arabidopsis* by INDEHISCENT, Cell 116 (2004) 843–853.
[62] E.A. Kellogg, Relationships of cereal crops and other grasses, Proc. Natl. Acad. Sci. USA 95 (1998) 2005–2010.
[63] E.A. Kellogg, Evolutionary history of the grasses, Plant Physiol. 125 (2001) 1198–1205.
[64] B. Zhong, T. Yonezawa, Y. Zhong, M. Hasegawa, Episodic evolution and adaptation of chloroplast genomes in ancestral grasses, PLoS ONE 4 (2009) e5297, doi:10.1371/journal.pone.0005297.

[65] S. Huang, A. Sirikhachornkit, X. Su, J. Faris, B. Gill, R. Haselkorn, P. Gornicki, Phylogenetic analysis of the acetyl-CoA carboxylase and 3-phosphoglycerate kinase loci in wheat and other grasses, Plant Mol. Biol. 48 (2002) 805–820.
[66] J. Dvořák, E.D. Akhunov, Tempos of gene locus deletions and duplications and their relationship to recombination rate during diploid and polyploid evolution in the Aegilops–Triticum alliance, Genetics 171 (2005) 323–332.
[67] S. Huang, A. Sirikhachornkit, X. Su, J. Faris, B. Gill, R. Haselkorn, P. Gornicki, Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the Triticum/Aegilops complex and the evolutionary history of polyploid wheat, Proc. Natl. Acad. Sci. USA 99 (2002) 8133–8138.
[68] A. Haudry, C. Cenci, C. Guilhaumon, E. Paux, S. Poirier, S. Santoni, J. David, S. Glemin, Mating system and recombination affect molecular evolution in four Triticeae species, Genet. Res. Camb. 90 (2008) 97–109.
[69] E. Paux, D. Roger, E. Badaeva, G. Gay, M. Bernard, P. Sourdille, C. Feuillet, Characterizing the composition and evolution of homoeologous genomes in hexaploid wheat through BAC-end sequencing on chromosome 3B, Plant J. 48 (2006) 463–474.
[70] R. Ragupathy, S. Cloutier, Genome organization and retrotransposon driven molecular evolution of the endosperm *Hardness* (*Ha*) locus in *Triticum aestivum* cv. Glenlea, Mol. Genet. Genomics 280 (2008) 467–481.